LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

UCRL-JRNL-235916

A Cell-Based Approach for the Biosynthesis/Screening of Cyclic Peptide Libraries against Bacterial Toxins

J. A. Camarero, R. Kimura, Y. Woo, J. Cantor, E. Steenblock

October 29, 2007

Chemistry Today

Disclaimer

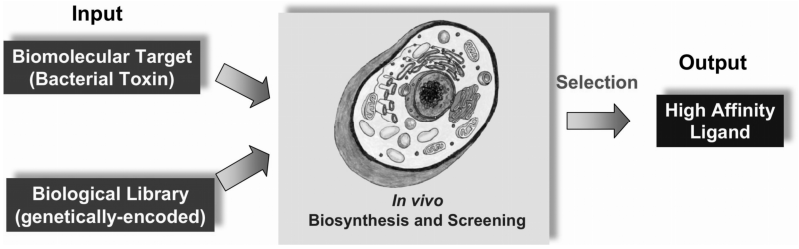
This document was prepared as an account of work sponsored by an agency of the United States government. Neither the United States government nor Lawrence Livermore National Security, LLC, nor any of their employees makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States government or Lawrence Livermore National Security, LLC. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States government or Lawrence Livermore National Security, LLC, and shall not be used for advertising or product endorsement purposes.

A Cell-Based Approach for the Biosynthesis/Screening of Cyclic Peptide Libraries against Bacterial Toxins

ABSTRACT
Available methods for developing and screening small drug-like molecules able to knockout toxins or pathogenic microorganisms have some limitations. In order to be useful, these new methods must provide high-throughput analysis and identify specific binders in a short period of time. To meet this need, we are developing an approach that uses living cells to generate libraries of small biomolecules, which are then screened inside the cell for activity. Our group is using this new, combined approach to find highly specific ligands capable of disabling anthrax Lethal Factor (LF) as proof of principle.
Key to our approach is the development of a method for the biosynthesis of libraries of cyclic peptides, and an efficient screening process that can be carried out inside the cell.

INTRODUCTION

The exposure of U.S. postal workers to *Bacillus anthracis,* the pathogen causing anthrax, in 2001 revealed a gap in the world's overall preparedness against bioterrorism. Unfortunately, anthrax is just one of many potential weapons of biochemical terrorism, there are so many other known toxins like ricin or botulinum [1] as well as unknown biological threats that could also be used against the civil population [2]. This alarming situation uncovers an urgent need for developing new methods for efficient synthesis, and fast screening of small high-affinity ligands able to bind and inactivate a particular biological toxin [3].
Available methods for producing and screening high-affinity inhibitors against particular molecular targets are either based in rational or combinatorial approaches [4]. The rational approach usually requires the molecular structure of the target to be knocked out, and then potential binders are selected from a virtual library of compounds using docking software [5]. Despite the recent advances in computing technology and development of adaptive docking software [5], this is still a slow although promising process. Combinatorial approaches, on the other hand, use a random approach to generate large number of compounds that are then screened against a biomolecular molecular target [6, 7] . Most of the methods for library screening, however, are performed *in vitro*, which is a long and laborious process.



Scheme 1. A cell-based approach for the biosynthesis and screening of genetically encoded libraries.

In vivo screening, on the other hand, opens the possibility of using single cells as microfactories where the biosynthesis and screening of particular inhibitor can take place in a single process within the same cellular cytoplasm (Scheme 1) [8]. The use of a complex molecular environment, such as the cellular cytoplasm, provides the ideal background for the selection of highly specific inhibitors. Furthermore, the recent introduction of genetically encoded fluorescence-based assays [9] allows the use high-throughput screening methods such as fluorescence-activated cell sorting (FACS) for studying molecular interactions inside living cells [10].

In this review I would like to present some of the most recent efforts of my group towards the development of a cell-based approach for the biosynthesis and high throughput screening of high affinity ligands against bacterial toxins inside living cells.

RESULTS

My group has been developing this combined approach to find highly specific ligands capable of disabling *Bacillus anthracis* toxins [11]. The high pathogenicity of anthrax is mostly due to rapid bacterial growth combined with the secretion of three powerful exotoxin components: edema factor (EF), lethal factor (LF), and protective antigen (PA). EF is a calcium and calmodulin-dependent adenylate cyclase (AC) that converts cellular ATP into cyclic AMP (cAMP) [12]. LF is a Zn^{2+}-dependent metalloprotease [13] that cleaves and inactivates mitogen-activated protein kinase kinases (MAPKKs) [14]. PA binds to a cell surface anthrax toxin receptor (ATR/TEM-8 or CMG-2) [15-17] where it is activated by proteolytic cleavage by furin-like proteases [18]. This step enables the formation of an heptameric pore [19] that allows cellular entry of LF and EF. Once inside the cell, LF and EF cause extensive cellular damage to the host cell defense system. Although the complete mechanism of pathogenesis is not yet fully understood, the disruption of key signaling pathways mediated by MAPKKs seems to lead first to the lysis of macrophages [20, 21], impairment of dendritic cells and later to the death of the host [22]. The pivotal role of LF in the virulence of the toxin suggests that inhibitors of this enzyme may provide protection against cytotoxicity. For this purpose we have recently developed an intein-based approach for the intracellular production of cyclic peptide libraries [11, 23] as well a new cell-based fluorescent assay for LF [11] that can be interfaced with FACS for the high throughput screening of potential LF inhibitors.

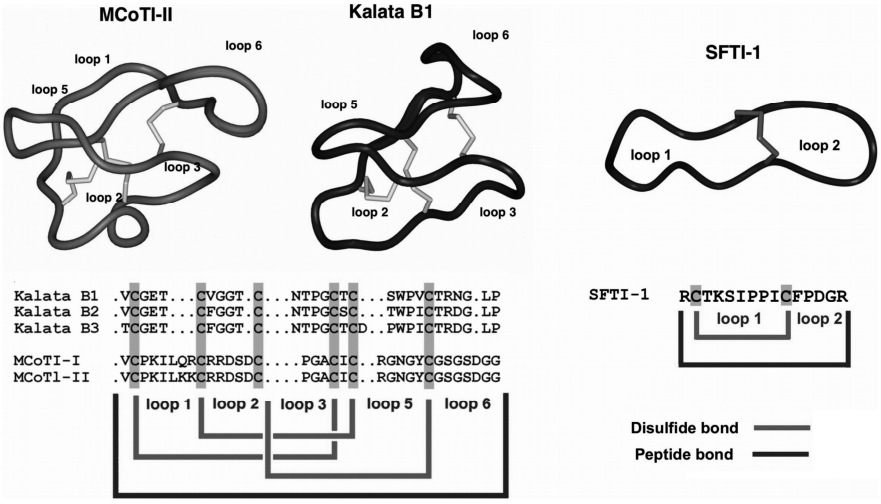


Figure 1. Primary and tertiary structure of cyclotides from the plants *Momordica cochinchinensis* (MCoTI-II) and *Oldenlandia affinis* (Kalata B1), and Bowman-Birk sunflower trypsin inhibitor 1 (SFTI-1).

In vivo biosynthesis of cyclotides and other cyclic peptides

Cyclotides are a new emerging family of large plant-derived backbone-cyclized polypeptides (≈28-37 amino acids long) that share a disulfide-stabilized core (3 disulfide bonds) characterized by an unusual knotted structure (Figure 1) [24, 25]. In this motif, an embedded ring formed by two disulfide bonds and their connecting backbone segments is penetrated by the third disulfide bond. Cyclotides contrast with other circular poylpeptides in that they have a well-defined three-dimensional structure, and despite their small size, can be considered as miniproteins. Their unique circular backbone topology and knotted arrangement of three disulfide bonds makes them exceptionally stable to thermal and enzymatic degradation [26]. Furthermore, their well-defined structures have been associated with a range of biological functions such as uterotonic activity, inhibition of trypsin and neurotension binding, cytotoxicity, anti-HIV, antimicrobial, and insecticidal activity [27, 28]. Together, these characteristics make cyclotides ideal candidates to be used as molecular scaffolds for the development of stable high affinity ligands in drug development [29, 30].

Our group has recently reported the use of engineered protein splicing units for the *in vitro* and *in vivo* generation of folded cyclotides [23]. Our biomimetic approach is based on the use of an intramolecular version of native chemical ligation (NCL) [31] to produce the head-to-tail or backbone cyclization of the corresponding linear precursor (Figure 2). Intramolecular NCL requires the presence of an N-terminal Cys residue and a C-terminal α-thioester group in the same linear precursor [32, 33]. As shown in Figure 2, this was accomplished by producing a recombinant protein where the linear cyclotide precursor was fused in frame at its C- and N-terminus to a modified intein and a Met

residue, respectively. This allows the generation of the required C-terminal α-thioester function [34] and N-terminal Cys residue [35]. This approach has been successfully used for the biosynthesis of the cyclotides Kalata B1 (KB1) [23] and MCoTI-II [11] as well as small libraries based on the cyclotide KB1 (Figure 3a and 3c) [23]. Using the same biosynthetic approach we have also produced a small library based on the Bowman-Birk inhibitor sunflower trypsin inhibitor-1 (SFTI-1) (Figure 3b and d) [11]. With just 14 amino acids SFTI-1 is the smallest and more potent naturally occurring Bowman-Birk inhibitor, and therefore it also provides an ideal template for the design of specific inhibitors to target proteases like LF [36, 37].

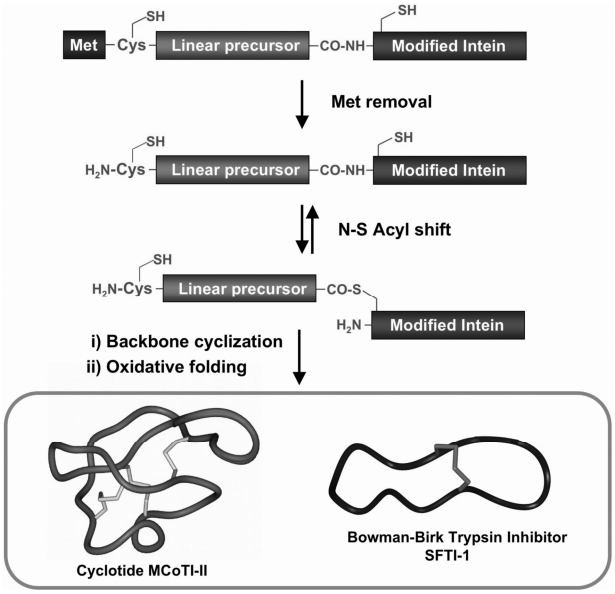


Figure 2. Biosynthetic approach for *in vivo* production of cyclotides KB1 and MCoTI-II, and Bowman-Birk trypsin inhibitor SFTI-1 inside live *E. coli* cells. Backbone cyclization of the linear precursor is mediated by a modified protein splicing unit or intein. The cyclized product then folds spontaneously in the bacterial cytoplasm.

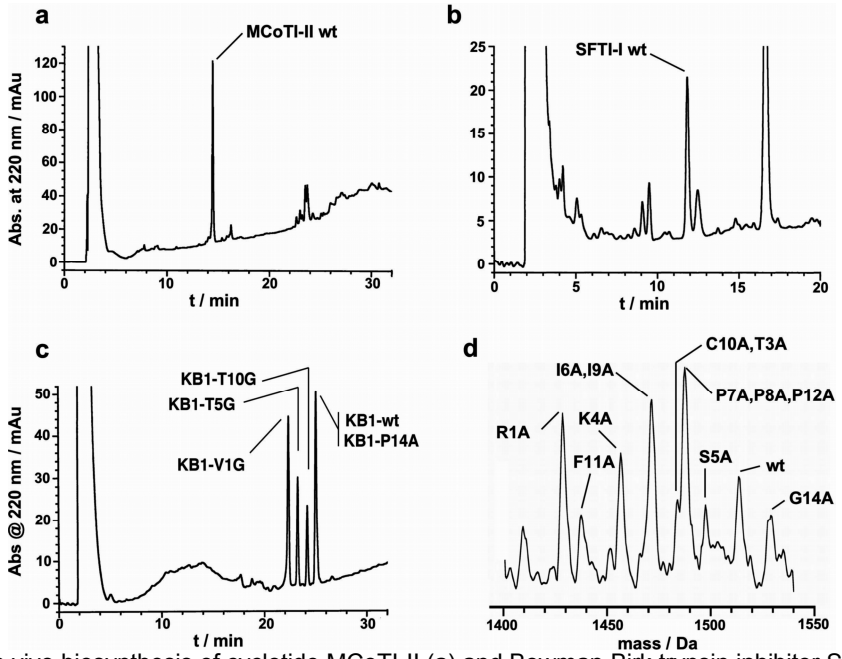


Figure 3. In vivo biosynthesis of cyclotide MCoTI-II (a) and Bowman-Birk trypsin inhibitor SFTI-1 (b) [11]. In both cases soluble cellular lysates were analyzed by reverse-phase HPLC after being purified by affinity chromatography on trypsin-immobilized agarose beads. Analytical HPLC trace of a KB1-based library (c) obtained *in vitro* under conditions mimicking physiological conditions [23]. ES-MS analysis of a SFTI-1 library encoding different Ala mutants (d) [11]. Numbering for KB1 and SFTI-1 mutants is as described in Figure 1.

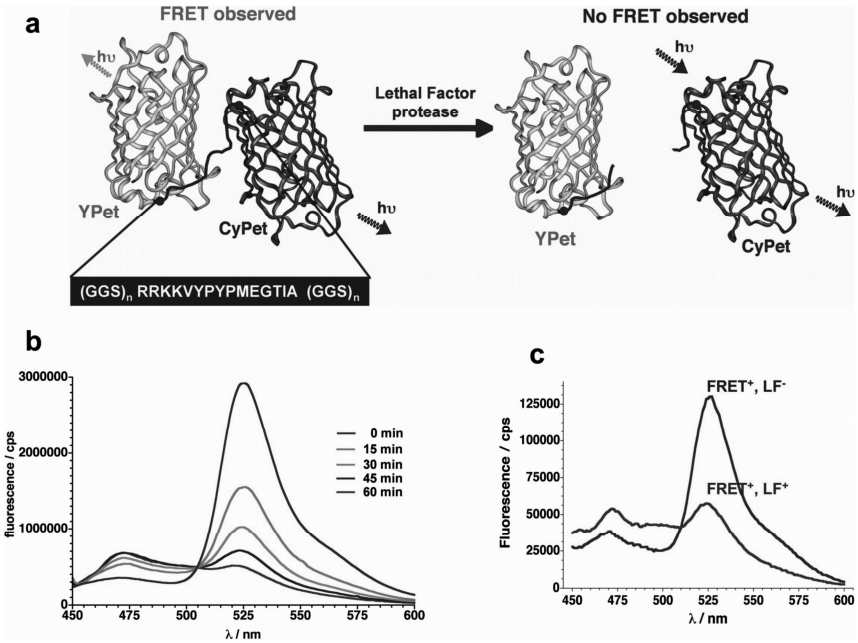


Figure 4. (a) Principle of a genetically encoded FRET reporter for LF activity. (b) *In vitro* cleavage of FRET reporter containing two Gly-Gly-Ser repeats by LF protease. Fluorescence spectra of a 10 nM solution of FRET reporter incubated with LF (100 nM) at different time points. Excitation was done at 413 nm. (c) *In vivo* cleavage of FRET reporter containing six Gly-Gly-Ser repeats followed by fluorescence spectroscopy. Fluorescence spectra of *E. coli* cells expressing LF reporter in the presence ($FRET^+$, LF^+) or absence ($FRET^+$, LF^-) of LF. B. Cells were excited at 490 nm.

Development of cell-based fluorescence resonance energy transfer (FRET) reporter for LF activity

We have also designed several genetically encoded FRET-based reporters specific for the anthrax LF protease. These reporters consist of an optimized FRET pair of fluorescent proteins, YPet and CyPet, which have been recently described by Daugherty and co-workers [38] for the study of molecular interactions *in vivo* [10]. The CyPet and YPet proteins were linked together by a flexible linker containing a consensus recognition site for LF protease [39] flanked at both its N- and C-termini by several repeats of the flexible tripeptide Gly-Gly-Ser (Figure 4a) [40]. *In vitro* evaluation of the different reporters showed that at least a minimum of two Gly-Gly-Ser repeats are required at each side of the LF peptide recognition motif for efficient cleavage by LF. Interestingly, the introduction of multiple Gly-Gly-Ser repeats in the linker between the CyPet and YPet fluorescent proteins had a relatively minor effect on the FRET efficiency of the protein constructs containing the longer linkers. *In vitro* evaluation of the different FRET reporters showed around a 10-fold decrease in FRET signal upon cleavage with LF (Figure 4b).

An optimized FRET-reporter containing six Gly-Gly-Ser repeats to each side of the LF peptide recognition motif was used next in the development of a cell-based FRET reporter for LF activity. This approach employs bacterial cells as individual micro-reactors where the substrate and the enzyme (i.e., LF protease) are sequentially expressed. This was accomplished by cloning the optimized FRET reporter and LF into two compatible, tightly controlled and inducible expression plasmids. The genetically encoded FRET-reporter was cloned into an expression pBAD-derived vector. This expression plasmid contains an araBAD-driven promoter and a p15 replicon. LF was cloned into a pRSF-based vector to give the expression plasmid pRSF-LF. This expression vector contains a T7-driven promoter and an RSF origin of replication [41]. These two expression plasmids are fully compatible for the sequential expression of proteins in *E. coli* cells, and they have been used for the study of protein–protein interactions *in vivo* [42].

We have recently shown that *E. coli* cells co-transformed with both plasmids can be induced into either the FRET-on or FRET-off states by expressing only the reporter protein or both sequentially, respectively (Figure 4c). The FRET signal of living bacteria induced to express the FRET-reporter decreased approximately 5 times in less than 1 hour upon induction of LF expression (Figure 4c). Hence, expression by the same cell of any potential LF inhibitor during the FRET-on state can be readily screened by measuring the FRET ratio at different times during the induction of LF. Potential inhibitors will inhibit the cleavage of the substrate at early times of LF induction. In contrast, cells containing non-inhibitors will efficiently cleave the substrate, rapidly reaching the FRET-off state.

CONCLUDING REMARKS

Recent advances in the fields of protein engineering are allowing for the first time the biosynthesis of complex cyclic peptides inside living cells [43]. We have recently used two natural disulfide-containing cyclic peptide scaffolds, the cyclotides Kalata B1 and MCoTI-II, and the Bowman-Birk SFTI-1 trypsin inhibitor (SFTI-1) as templates for the *in vivo* biosynthesis of peptide libraries using protein splicing units [11, 23]. These types of scaffolds have a tremendous potential for the development of therapeutic leads based on their extraordinary stability and potential for grafting applications [36, 44]. We have also developed a cell-based FRET reporter for anthrax LF protease that can be easily interfaced with sensitive FACS techniques for the rapid screening of genetically-encoded peptide-based libraries in *E. coli* against LF.

It is also important to remark that although our initial efforts have focused is the production of high-affinity ligands that can disable bacterial toxins, this cell-based approach can also be easily used for screening molecules capable of disrupting any biomolecular interaction. For example, this approach could be also used to find molecules that may disrupt the destructive mechanisms involved in cancer and neurodegenerative diseases such mad cow and Alzheimer's.

Acknowledgements

We would like to thank Dr. Alexander R. Mitchell for useful discussions. This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344

REFERENCES AND NOTES

[1] R. P. Hicks, M. G. Hartell, D. A. Nichols, A. K. Bhattacharjee, J. E. van Hamont, and D. R. Skillman, *Curr. Med. Chem.* The medicinal chemistry of botulinum, ricin and anthrax toxins Vol. 12, 667-90 (2005).

[2] R. A. Greenfield and M. S. Bronze, *Drug Discov. Today* Prevention and treatment of bacterial diseases caused by bacterial bioterrorism threat agents Vol. 8, 881-8 (2003).

[3] G. J. Rainey and J. A. Young, *Nat. Rev. Microbiol.* Antitoxins: novel strategies to target agents of bioterrorism Vol. 2, 721-6 (2004).

[4] L. O. Haustedt, C. Mang, K. Siems, and H. Schiewe, *Curr. Opin. Drug Discov. Devel.* Rational approaches to natural-product-based drug design Vol. 9, 445-62 (2006).

[5] B. Waszkowycz, *Curr. Opin. Drug Discov. Devel.* Structure-based approaches to drug design and virtual screening Vol. 5, 407-13 (2002).

[6] K. S. Lam, M. Lebl, and V. Krchnak, *Chem. Rev.* The "One-Bead-One-Compound" Combinatorial Library Method Vol. 97, (1997).

[7] T. Clackson, H. R. Hoogenboom, A. D. Griffiths, and G. Winter, *Nature* Making antibody fragments using phage display libraries Vol. 352, 624-8 (1991).

[8] I. Kaganman, *Nat. Methods* FRETting for a more detailed interactome Vol. 4, 112-3 (2007).

[9] B. N. Giepmans, S. R. Adams, M. H. Ellisman, and R. Y. Tsien, *Science* The fluorescent toolbox for assessing protein location and function Vol. 312, 217-24 (2006).

[10] X. You, A. W. Nguyen, A. Jabaiah, M. A. Sheff, K. S. Thorn, and P. S. Daugherty, *Proc. Natl. Acad. Sci. U S A* Intracellular protein interaction mapping with FRET hybrids Vol. 103, 18458-63 (2006).

[11] J. A. Camarero, R. Kimura, and A. T. Tran, *J. Pept. Sci.* A Cell-based Approach for Biosynthesis/Screening of Cyclic Peptide Libraries against Bacterial Toxins Vol. 12, 89 (2006).

[12] S. H. Leppla, *Proc. Natl. Acad. Sci. U S A* Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells Vol. 79, 3162-6 (1982).

[13] K. R. Klimpel, N. Arora, and S. H. Leppla, *Mol. Microbiol.* Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity Vol. 13, 1093-100 (1994).

[14] C. Montecucco, F. Tonello, and G. Zanotti, *Trends Biochem. Sci.* Stop the killer: how to inhibit the anthrax lethal factor metalloprotease Vol. 29, 282-5 (2004).

[15] K. A. Bradley, J. Mogridge, M. Mourez, R. J. Collier, and J. A. Young, *Nature* Identification of the cellular receptor for anthrax toxin Vol. 414, 225-9 (2001).

[16] H. M. Scobie, G. J. Rainey, K. A. Bradley, and J. A. Young, *Proc. Natl. Acad. Sci. U S A* Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor Vol. 100, 5170-4 (2003).

[17] E. Santelli, L. A. Bankston, S. H. Leppla, and R. C. Liddington, *Nature* Crystal structure of a complex between anthrax toxin and its host cell receptor Vol. 430, 905-8 (2004).

[18] V. M. Gordon, K. R. Klimpel, N. Arora, M. A. Henderson, and S. H. Leppla, *Infect. Immun.* Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases Vol. 63, 82-7 (1995).

[19] J. C. Milne, D. Furlong, P. C. Hanna, J. S. Wall, and R. J. Collier, *J. Biol. Chem.* Anthrax protective antigen forms oligomers during intoxication of mammalian cells Vol. 269, 20607-12 (1994).

[20] J. M. Park, F. R. Greten, Z. W. Li, and M. Karin, *Science* Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition Vol. 297, 2048-51 (2002).

[21] J. E. Kirby, *Infect. Immun.* Anthrax lethal toxin induces human endothelial cell apoptosis Vol. 72, 430-9 (2004).
[22] A. Agrawal, J. Lingappa, S. H. Leppla, S. Agrawal, A. Jabbar, C. Quinn, and B. Pulendran, *Nature* Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin Vol. 424, 329-34 (2003).
[23] R. H. Kimura, A. T. Tran, and J. A. Camarero, *Angew. Chem. Int. Ed.* Biosynthesis of the cyclotide kalata B1 by using protein splicing Vol. 45, 973-6 (2006).
[24] D. J. Craik, N. L. Daly, T. Bond, and C. Waine, *J. Mol. Biol.* Plant cyclotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif Vol. 294, 1327-36 (1999).
[25] K. J. Rosengren, N. L. Daly, M. R. Plan, C. Waine, and D. J. Craik, *J. Biol. Chem.* Twists, knots, and rings in proteins. Structural definition of the cyclotide framework Vol. 278, 8606-16 (2003).
[26] M. L. Colgrave and D. J. Craik, *Biochemistry* Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot Vol. 43, 5965-75 (2004).
[27] U. Goransson, E. Svangard, P. Claeson, and L. Bohlin, *Curr. Protein Pept. Sci.* Novel strategies for isolation and characterization of cyclotides: the discovery of bioactive macrocyclic plant polypeptides in the Violaceae Vol. 5, 317-29 (2004).
[28] D. J. Craik, N. L. Daly, J. Mulvenna, M. R. Plan, and M. Trabi, *Curr. Protein Pept. Sci.* Discovery, structure and biological activities of the cyclotides Vol. 5, 297-315 (2004).
[29] D. J. Craik, M. Cemazar, C. K. Wang, and N. L. Daly, *Biopolymers* The cyclotide family of circular miniproteins: nature's combinatorial peptide template Vol. 84, 250-66 (2006).
[30] D. J. Craik, M. Cemazar, and N. L. Daly, *Curr. Opin. Drug Discov. Devel.* The cyclotides and related macrocyclic peptides as scaffolds in drug design Vol. 9, 251-60 (2006).
[31] P. E. Dawson and S. B. Kent, *Annu. Rev. Biochem.* Synthesis of native proteins by chemical ligation Vol. 69, 923-60 (2000).
[32] J. A. Camarero, J. Pavel, and T. W. Muir, *Angew. Chem. Int. Ed.* Chemical Synthesis of a Circular Protein Domain: Evidence for Folding-Assisted Cyclization Vol. 37, 347-349 (1998).
[33] J. A. Camarero and T. W. Muir, *J. Am. Chem. Soc.* Biosynthesis of a Head-to-Tail Cyclized Protein with Improved Biological Activity Vol. 121, 5597-5598 (1999).
[34] T. W. Muir, *Annu. Rev. Biochem.* Semisynthesis of proteins by expressed protein ligation Vol. 72, 249-289 (2003).
[35] J. A. Camarero, D. Fushman, D. Cowburn, and T. W. Muir, *Bioorg. Med. Chem.* Peptide chemical ligation inside living cells: in vivo generation of a circular protein domain Vol. 9, 2479-84. (2001).
[36] M. L. Korsinczky, H. J. Schirra, and D. J. Craik, *Curr. Protein Pept. Sci.* Sunflower trypsin inhibitor-1 Vol. 5, 351-64 (2004).
[37] R. F. Qi, Z. W. Song, and C. W. Chi, *Acta Biochim. Biophys. Sin (Shanghai)* Structural features and molecular evolution of Bowman-Birk protease inhibitors and their potential application Vol. 37, 283-92 (2005).
[38] A. W. Nguyen and P. S. Daugherty, *Nat. Biotechnol.* Evolutionary optimization of fluorescent proteins for intracellular FRET Vol. 23, 355-60 (2005).
[39] B. E. Turk, T. Y. Wong, R. Schwarzenbacher, E. T. Jarrell, S. H. Leppla, R. J. Collier, R. C. Liddington, and L. C. Cantley, *Nat. Struct. Mol. Biol.* The structural basis for substrate and inhibitor selectivity of the anthrax lethal factor Vol. 11, 60-6 (2004).
[40] T. H. Evers, E. M. van Dongen, A. C. Faesen, E. W. Meijer, and M. Merkx, *Biochemistry* Quantitative understanding of the energy transfer between fluorescent proteins connected via flexible peptide linkers Vol. 45, 13183-92 (2006).
[41] T. Som and J. Tomizawa, *Mol. Gen. Genet.* Origin of replication of Escherichia coli plasmid RSF 1030 Vol. 187, 375-83 (1982).
[42] D. S. Burz, K. Dutta, D. Cowburn, and A. Shekhtman, *Nat. Methods* Mapping structural interactions using in-cell NMR spectroscopy (STINT-NMR) Vol. 3, 91-3 (2006).
[43] R. Kimura and J. A. Camarero, *Protein Pept. Lett.* Expressed protein ligation: a new tool for the biosynthesis of cyclic polypeptides Vol. 12, 789-94 (2005).
[44] D. J. Craik, S. Simonsen, and N. L. Daly, *Curr. Opin. Drug Discov. Devel.* The cyclotides: novel macrocyclic peptides as scaffolds in drug design Vol. 5, 251-260 (2002).

JULIO A. CAMARERO*, RICHARD H. KIMURA, YOUN-HI WOO, JASON CANTOR AND ERIN STEENBLOCK
*Corresponding author
Lawrence Livermore National Laboratory, University of California
7000 East Avenue, L-231, Livermore, CA 94550, USA
Phone: (925) 422 6807
Fax: (925) 422 3160
E-mail: camarero1@llnl.gov